

Identification of a Cytotoxic T-Cell Epitope on the Recombinant Nucleocapsid Proteins of Rinderpest and *Peste des petits ruminants* Viruses Presented as Assembled Nucleocapsids

Shibani Mitra-Kaushik, Rabindranath Nayak, and M. S. Shaila¹

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India

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The nucleocapsid protein (N) of morbilliviruses is not only a major structural protein but also the most abundant protein made in infected cells. We overexpressed the N proteins of Rinderpest virus and *Peste des petits ruminants* virus in *E. coli*, which assemble into nucleocapsids in the absence of viral RNA that resemble nucleocapsids made in the virus-infected cells. Employing these assembled structures resembling subviral particles, we studied the induction of both the antibody response and the cytotoxic T-lymphocyte (CTL) response in a murine model (BALB/c). A single dose of the purified recombinant nucleocapsids of both viruses in the absence of an adjuvant induces a strong CTL response. The CTLs generated are antigen specific and cross-reactive with respect to each virus and, furthermore, this CTL response is MHC class I restricted. Based on the prediction for H-2^d-restricted T-cell motifs we tested the lysis of transfected P815 (H-2^d) cells expressing a nine amino acid potential CTL epitope, by splenic T cells *in vitro* restimulated with bacterially expressed RPV or PPRV N proteins. We extended our study to the bovine system both to analyze the immunogenicity of these recombinant proteins in the natural hosts and to show that PBMC from cattle vaccinated with Rinderpest vaccine proliferate *in vitro*, in response to restimulation with soluble nucleocapsid proteins. Furthermore, the murine CTL epitope functions in the bovine system as a cytotoxic T-cell epitope. This sequence, which is conserved in the N proteins of morbilliviruses, conforms well to the predicted algorithm for some of the most common BoLA CTL antigenic peptides. © 2001 Academic Press

INTRODUCTION

Rinderpest virus (RPV) and *Peste des petits ruminants* virus (PPRV) are important members of the *Morbillivirus* genus in the *Paramyxoviridae* family and cause acute, febrile, and contagious viral disease in large and small ruminants, both domestic and wild. The RNA genome of these viruses is associated with the nucleocapsid protein N in the virion and throughout the viral replicative cycle as a filamentous ribonucleoprotein complex with a characteristic herringbone appearance. Although the actual nature of the host-immune response to the infection is largely unknown, the fact that there is severe immunosuppression accompanying a massive virus-specific immune response is an intriguing paradox (Randall and Russell, 1991; Griffin, 1995; Morrison, 1995). Surface glycoproteins H (hemagglutinin) and F (fusion protein) induce strong protective virus-neutralizing antibodies (Romero *et al.*, 1994a,b, 1995), while the cell-mediated immune response elicited by these proteins aids in virus clearance (Norrby, 1991). Internal proteins such as the nucleoprotein N have been found to induce strong cell-mediated immune responses, which aid in protective immunity and, in the case of measles virus (MV), these N-specific T cells constitute a major fraction of the

virus-specific memory cells (Griffin, 1995; Hickman *et al.*, 1997).

Adenovirus-5 recombinant expressing MV N protein was used to study potential CTL epitope in mouse models (Schadeck *et al.*, 1999). One of the H-2^d-restricted epitopes and an antigenically related morbillivirus canine distemper virus (CDV) were found to be protective against MV. Also, a DNA vaccine encoding the nucleocapsid and surface glycoproteins of wild type CDV was found to protect its natural hosts against distemper (Cherpillod *et al.*, 2000). Earlier studies with a recombinant baculovirus and adenovirus recombinants expressing RPV H and F genes demonstrated the generation of proliferative T-cell responses, although no CTL activity could be detected (Bassiri *et al.*, 1993). A recombinant vaccinia virus expressing the RPV N protein was shown to generate protective immunity upon challenge with mildly virulent challenge virus, although protection was not observed in challenge experiments with a highly virulent strain (Ohishi *et al.*, 1999). Studies in the rabbit model with a vaccinia virus recombinant expressing the nucleoprotein of the Rinderpest virus indicate that the N protein administered via such routes was ineffective in priming protective immunity and, at best, can only delay the onset of the disease in immunized animals (Nakamura *et al.*, 1998). Since morbilliviruses are cytopathic viruses, an ideal subunit vaccine should include a suitable combination of B-cell epitopes eliciting strong virus-

¹ To whom correspondence and reprint requests should be addressed. Fax: 91-80-3602697. E-mail: shaila@mcbl.iisc.ernet.in.

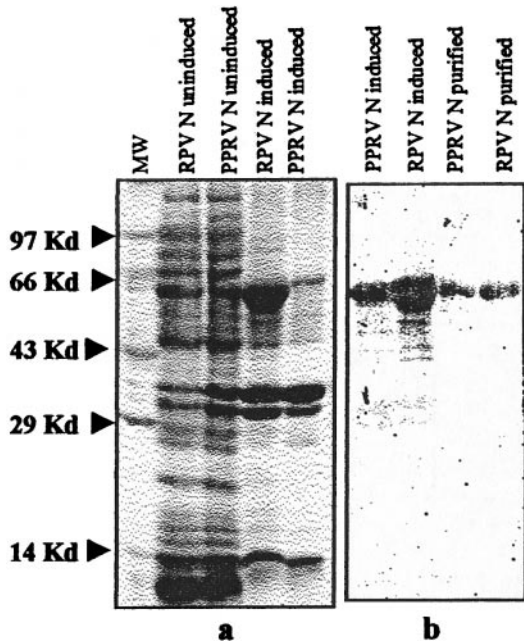


FIG. 1. Purification of bacterially expressed RPV and PPRV N proteins. The cDNA coding for the RPV and PPRV nucleocapsid proteins were expressed from pET20b and pRSET B respectively in the *E. coli* BL21(DE3). Expression from the T7 promoter was induced by 0.4 mM IPTG for 4 h, purified to near homogeneity by ultracentrifugation on a CsCl gradient, and electrophoresed on a 10% SDS-PAGE. The authenticity of the expressed protein and the purity was checked by transfer onto a nitrocellulose membrane and probing the blot with anti-N antibody raised against the purified viral nucleocapsids.

neutralizing antibodies, such as those present on the H and F proteins, and CTL epitopes used in infected hosts, predominantly from the internal proteins such as the N protein. Soluble antigens, however, very rarely generate CTL responses (Forquet *et al.*, 1990; Gromme *et al.*, 1999) and are a major hindrance in the administration of recombinant proteins as vaccines. Previous studies in other viruses showed that recombinant nucleocapsid proteins assemble into viral nucleocapsid-like structures and can be visualized as assembled nucleocapsids by electron microscope (Coronel *et al.*, 1999; Fooks *et al.*, 1993, 1996; Spehner *et al.*, 1991). Such assembled nucleocapsid particles were reported to elicit strong virus-specific CTL responses (Fooks *et al.*, 1995; Fu *et al.*, 1991; Lo-Man *et al.*, 1998; Marais *et al.*, 1999; Nakamura *et al.*, 1998; Rudolf *et al.*, 1999; Sedlik *et al.*, 1999).

We expressed the RPV N and PPRV N proteins in *E. coli* and the soluble recombinant N proteins assemble into nucleocapsid-like structures in the absence of viral RNA (unpublished results). In the present work, we studied the immune responses to these assembled nucleocapsids in the BALB/c mouse model, induced by a single low dose of immunization without any adjuvant or booster administration. Based on structural predictions and homology to a highly conserved immunodominant epitope of measles virus N protein (Beauverger *et al.*,

1993, 1996), oligonucleotide sequences corresponding to a nine amino acid CTL epitope were designed and cloned in a prokaryotic vector. This was moved into a eukaryotic vector along with 5' extra sequences harboring the initiator codon, to facilitate transient expression of the CTL epitope. Using this construct, we identified the CTL epitope on N protein in the BALB/c mouse, which is (H-2^d) restricted. We used a similar approach to map this sequence as a BoLA (bovine leucocyte antigen)-restricted cattle CTL epitope in cattle.

RESULTS

Recombinant RPV and PPRV nucleocapsid proteins induce proliferation of immune lymphocytes *in vitro*

Recombinant RPV and PPRV nucleocapsid proteins were expressed in *E. coli* and purified by density gradient ultracentrifugation. Figure 1 shows the purity of the protein ascertained by SDS-PAGE and Western blot. The purified proteins were further subjected to electron microscopy and were seen as assembled nucleocapsids very similar to the assembled structure of the viral nucleocapsids as shown in Fig. 2. Such assembled structures were used to immunize mice and the immune responses studied in these immune BALB/c mice. The proliferative responses of splenocytes from immune mice were found to be dose dependent (data not shown) and were optimum at an antigenic dose of 2 μ g/well, as seen in Fig. 3. The incubation of the cells in the presence of anti-murine class II antibodies was found to inhibit the proliferation, although the inhibition was not complete. This indicated the expansion of T cells by mechanisms other than that of either MHC II-CD4⁺ interactions or, more simply, the activation of the CD8⁺ T cells in response to MHC presentation of endogenously processed peptides from recombinant RPV and PPRV nucleocapsid proteins.

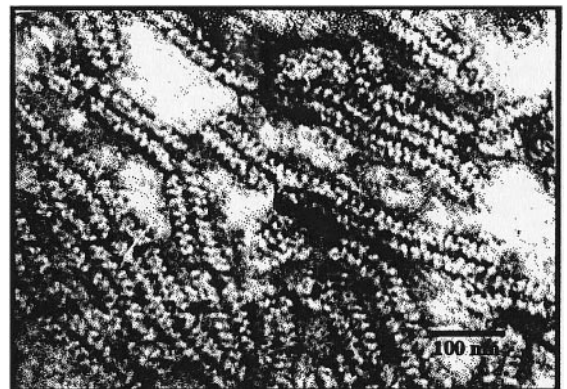


FIG. 2. Electron microscopy of the assembled recombinant nucleocapsids. Purified nucleocapsid proteins expressed in *E. coli* BL21(DE3) were resuspended in PBS at a concentration of 250 μ g/ml. The grid was examined in a Jeol 100cX11 electron microscope at 80 kV at a magnification of 100,000 \times .

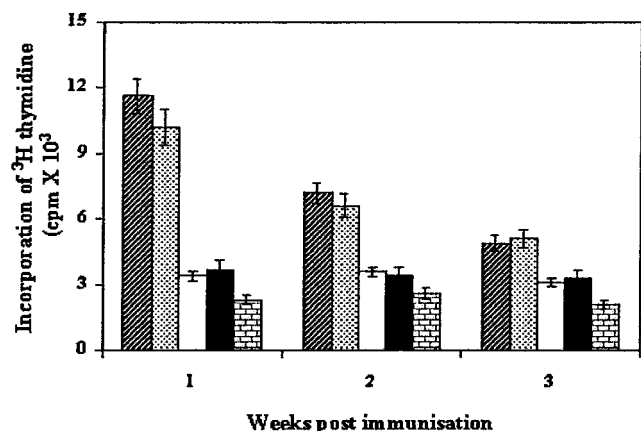


FIG. 3. *In vitro* proliferation of BALB/c immune T cells in response to recombinant nucleocapsid proteins. Lymphocytes were prepared from spleen of immune animals 3 weeks after immunization and further purified through Ficoll-Hypaque. Lymphocytes (1×10^4) were plated in 96-well plates with either RPV N (▨), PPRV N (▤) at different concentrations, RPV N + anti-MHC II antibodies (□), PPRV N + anti-MHC II (▧) antibodies, or without any antigen (▩) and incubated for 5 days. Triplicate samples of cells were labeled with [3 H]thymidine and the incorporated radioactivity was measured.

Generation of MHC class I-restricted CTL activity in immune BALB/c lymphocytes

Effector cells were prepared by the restimulation of isolated immune lymphocytes by the recombinant N proteins *in vitro* for 5 days and incubated with syngenic P815 targets. The specific stimulated effectors show up to 60% specific lysis at an E:T ratio of 80:1 with homologous targets as well as cross-reactive targets, i.e., RPV N immune effectors cocultured with PPRV N expressing P815 cells, depicted in Fig. 4A. Similarly PPRV N immune effectors brought about the specific lysis of both homologous and cross-reactive targets. The percentage specific lysis in this case was up to 50% at an E:T ratio of 80:1, as shown in Fig. 4B. The MHC I restriction of the CTL lysis was seen from inhibition of the lysis by the anti-MHC class I antibodies in the case of both immune effectors. The lysis by lymphocytes from control animals was subtracted to represent antigen-specific lysis.

Epitope-specific CTL responses in mice immunized with the recombinant N protein expressed as assembled nucleocapsids

The predicted measles virus CTL epitope was found to be conserved in both RPV and PPRV N proteins, and we derived both H-2^d and BoLA binding predictions (Hegde *et al.*, 1995, 1999; Hegde and Srikumaran, 1996) for this sequence (Table 1). We cloned the synthetic minigene harboring this sequence for CTL epitope in a eukaryotic expression vector (Fig. 5) and the minigene construct was used to transfect P815 cells which were subsequently used as targets in a typical CTL assay. Figure 6 shows the results of this assay, in which the lysis of the

epitope-expressing targets appears to be similar to that of the targets expressing the full-length RPV N protein, indicating that this epitope may be one of the immunodominant epitopes in the H-2^d-restricted animals. As expected, the lysis was abrogated by preincubation of the targets with anti-MHC class I antibodies, thus confirming the authenticity of this predicted nine amino acid sequence as a CTL epitope.

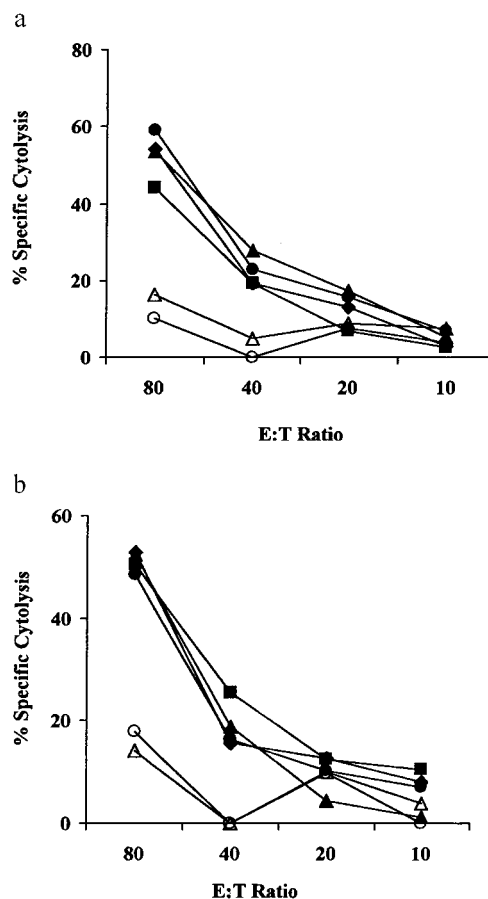


FIG. 4. (a) CTL assay using RPVN immunized BALB/c lymphocytes stimulated *in vitro* with recombinant RPVN or PPRV N. Lymphocytes were isolated from immune animals 3 weeks postimmunization and restimulated *in vitro* with the respective N proteins for 5 days. A CTL assay was performed in 96-well plates using RPV-infected P815 targets (▲), PPRV-infected P815 targets (■), RPV-infected targets + anti-MHC I antibodies (△), RPV N stable transfected targets (●), PPRV N stable transfected targets (◆), and RPV N stable transfected targets pre-treated with anti-MHC I antibodies (○). The percentage specific lysis was calculated according to the manufacturer's directions and is the mean of triplicate wells. (b) CTL assay using PPRVN-immunized BALB/c lymphocytes. Isolated PPRV N immune, murine splenic lymphocytes harvested 3 weeks postimmunization were restimulated *in vitro* with *E. coli* expressed and purified PPRV N or RPV N and used in a CTL assay with targets as follows: PPRV-infected P815 targets (▲), RPV-infected P815 targets (■), PPRV-infected targets + anti-MHC I antibodies (△), PPRV N stable transfected targets (●), RPV N stable transfected targets (◆), and PPRV N stable transfected targets pre-treated with anti-MHC I antibodies (○).

TABLE 1

Comparison of the Conserved Nine Amino Acid Sequence with the Peptide Motif of the Cattle MHC Class I Antigen BoLA A-11

Allele	1	2	3	4	5	6	7	8	9	10	11	12
BoLA A-11 ^a	X	P	<u>A</u>	I	<u>P</u>	V	L	Q	I	Y	A	V
		1	F	P	G	I	I	V	V			
			V	M	A	K	V	F	L			
			N	L	Y	Q	Y	I	Y			
			K	E	m	L	P	E				
			Y	G	q	G	G	Y				
			L	T	h	A	r	a				
			T	n	d	E	k	w				
			G	w	e		d	r				
			R	c				s				
			E					t				
			S									
			D									
			i									
			m									
			q									
RPV N aa 281-289	Y	P	A	L	G	L	H	E	F			
PPRV N aa 281-289	Y	P	A	L	G	L	H	E	F			

Note. Positional characteristics for BoLA A-11-bound peptides were compared. The numbers indicate the positions in the peptides eluted from BoLA A-11 antigen, and the amino acids are represented in single-letter codes. The descending order of amino acids approximately corresponds to the strength of the signal. Lowercase letters indicate weak anchors; bold letters indicate dominant anchors; underlined letters indicate possible auxiliary anchors. The conserved nine amino acids of the two viruses are compared for possible presentation on BoLA antigens.

^a See Hickman *et al.* (1997)

MHC class I-biased proliferation of PBMC from RPV-vaccinated cattle

To study the immunological properties of the proliferating T cells as seen in this study we demarcated the functional responders with respect to their CD4⁺ and the CD8⁺ T-cell nature, especially since the response is to a classical internal viral protein, which is normally presented to CTLs in the context of MHC I presentation. Skin fibroblast cells were previously shown to be MHC I⁺ and MHC II⁻ and, therefore, proliferation induced by such antigen-presenting cells is mostly the result of antigen presentation by the MHC class I pathway (Ohishi *et al.*, 1999). We used a similar assay to define the T-cell responses to the RPV N and the PPRV N, as well as the CTL epitope, by coculturing the immune PBMC after *in vitro* restimulation with the irradiated autologous skin cells of the bull transiently expressing the full-length RPV N or the PPRV N or the CTL epitope from the transfected DNA constructs. The observed proliferation was plotted in Fig. 7 and is expected to be represented in majority by the CD8⁺ T-cell proliferations. The stimulation index in this assay was found to be between 5 and 6 and the proliferation values for the CTL epitope matched that of the full-length RPV N or PPRV N, as was also observed in

the murine model. This confirms our observation that this nine amino acid sequence is indeed a CTL epitope for the BoLA restriction in the single vaccinated bull that we used in this study. Although this epitope needs to be tested in a more significant number of cattle, our findings suggest the definitive contribution of this epitope in the RPV N-specific CTL response.

CTL responses to the recombinant RPV N and PPRV N proteins in the bovine model

The N-specific CTL response was determined by the lysis of RPV N, PPRV N, or CTL epitope expressing autologous skin fibroblast targets. Untransfected skin cells were used as negative controls in the CTL assays. Effectors were generated by *in vitro* restimulation of the vaccinated PBMC by RBOK-RPV- or PPRV Nigeria 75/1-infected autologous skin cells for 5 days. We used PPRV-infected cells as a cross-reactive PPRV antigen-presenting cell and, interestingly, we find good levels of specific lysis with both of the effector populations, as shown in Fig. 8. It is expected that the conservation of the sequence in these related viruses would ensure the lysis of the PPRV targets, if there indeed were any T cells directed to the conserved cross-reactive epitopes on both proteins. The results conclusively show that the nine amino acid BoLA CTL epitope, which is conserved in almost all morbilliviruses, is also a dominant epitope in the bovine system. The detection of N-specific CTL after a secondary response in a natural host lends support to a further characterization of this protein for candidate epitopes in a subunit vaccine.

DISCUSSION

The most important observation in this study is that bacterially expressed recombinant RPV and PPRV nucleocapsid proteins elicit strong CD8⁺ T-cell responses, detectable both in BALB/c mice and in the target animal. The work confirms the earlier observation from other laboratories that N protein-immune CD4⁺ and CD8⁺ T cells proliferate *in vitro* when restimulated with both recombinant assembled nucleocapsids as well as MHC I-bound processed peptides on transfected or infected antigen-presenting cells (Forquet *et al.*, 1990; Jondal *et al.*, 1996; Sedlik *et al.*, 1999). The recombinant N proteins were found to generate high titers of antibodies that are not virus neutralizing, indicating the immunogenicity of such recombinant protein structures used as virus-like particles. Spleens of BALB/c mice immunized with purified recombinant RPV nucleocapsid or PPRV nucleocapsid proteins showed an increase in their dry weight at the end of 3 weeks (data not shown), indicating high levels of proliferation of splenic lymphocytes. The immune mice developed N-specific antibody titers of up to 1:16,000 at 2 weeks after immunization and the levels of antibody were maintained until 3 weeks postimmuniza-

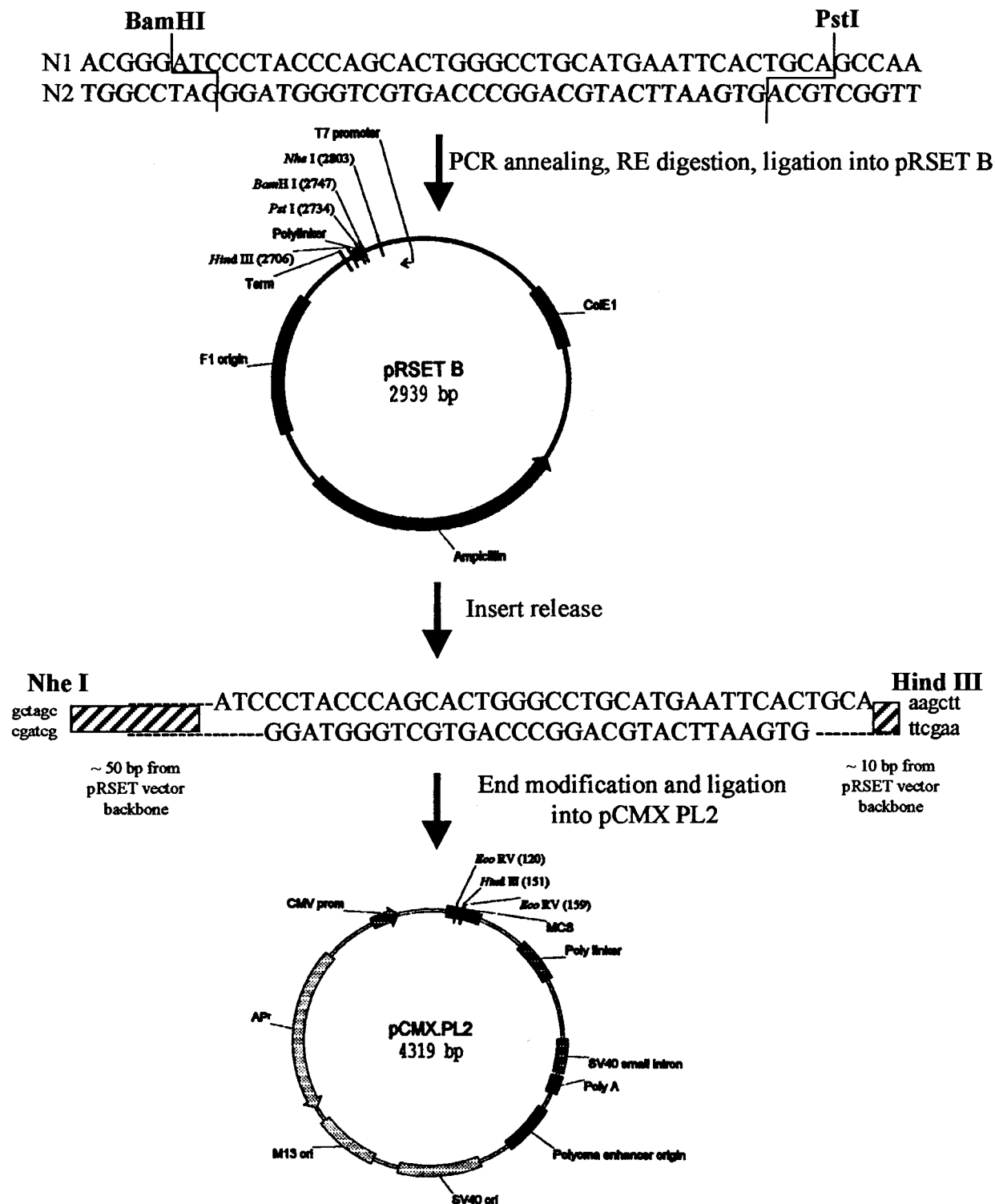


FIG. 5. Schematic representation of the generation of the synthetic minigene construct harboring the N CTL epitope for expression in eukaryotic cells.

tion (p.i.) at similar levels (data not shown). The antibodies were cross-reactive with respect to the two antigens used in this study (data not shown). Although the N-specific antibodies are not expected to be of any use in the clearance of infected cells or of cell-free virus parti-

cles, the high antibody levels indicate an ongoing response to the immunization mediated by T-cell help.

CD4⁺ cells generated in these responses not only play a role in the activation of virus-specific CTLs but also help to produce virus-specific B cells in a secondary

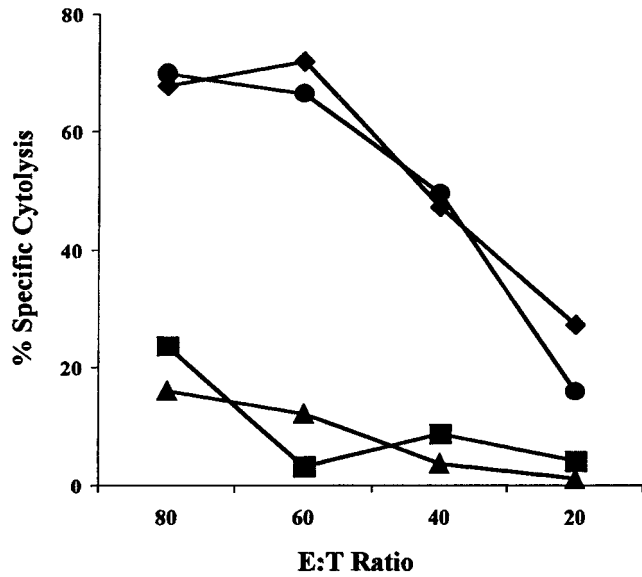


FIG. 6. CTL lysis of target cells expressing the N CTL epitope. P815 cells were transfected with the minigene construct expressing the nine amino acid CTL epitope and used as targets in a standard LDH release cytotoxicity assay. Untransfected P815 cells were also included as negative target to determine the background or nonspecific lysis of the assay conditions. The immune effectors obtained from RPV N and, PPRV N immune mice splenocytes were coincubated with the following target cells: RPV N (◆), CTL construct (●), P815 cells (▲), and CTL construct + anti-MHC I antibodies (■) for 16 h.

response. Quite notably the N protein-specific T cells were previously found to comprise the bulk of the virus-specific memory cells in the paramyxovirus family (Karp *et al.*, 1996). Since RPV and PPRV are cytopathic viruses such activated CD8⁺ T cells are unlikely to be involved in the acute stage of the disease, since here the control is more likely to be mediated by neutralizing antibodies. At

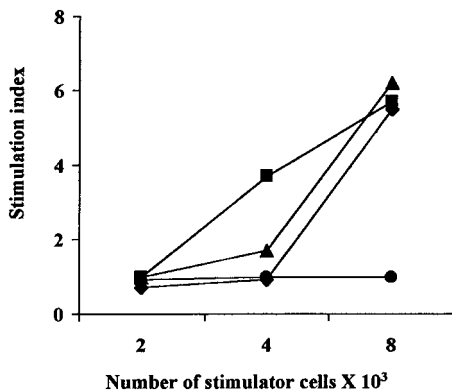


FIG. 7. Class I-biased proliferation of vaccinated cattle PBMC. Autologous skin fibroblasts transfected with the RPV N gene (◆), PPRV N gene (■), CTL epitope (▲), or vector-transfected cells (●) were used in a proliferation assay against the immune bovine PBMC in a proliferation assay performed 28 weeks postimmunization at different cell densities of the stimulator cells. Incorporation of the tritiated thymidine by the proliferating bovine lymphocytes was measured 5 days after restimulation.

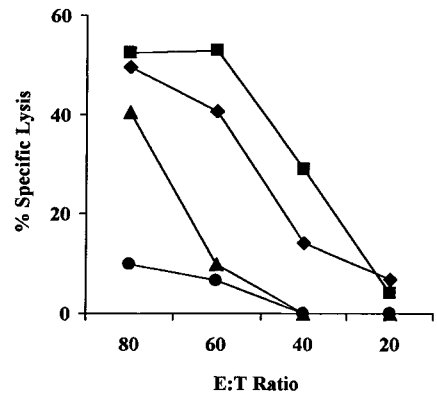


FIG. 8. Cytotoxic T-cell activity of PBMC from vaccinated cattle. CTL assay in the bovine system was performed similarly to that of the MHC I-biased CTL assay, except that the effector cells were restimulated *in vitro* for 5 days with the RPV- or PPRV-infected autologous skin fibroblast cells before the CTL assay was set up. Subsequently, the effector cells were harvested and plated with the targets, RPV N transfected fibroblast (◆), PPRV N transfected fibroblasts (■), and CTL epitope transfected cells (▲), and vector DNA transfected control cells (●) at different effector:target ratios for the CTL assays.

later stages, however, particularly during the establishment of infection, when these CD8⁺ T cells are known to proliferate, it is possible that they may play an as yet undefined role in the immunosuppression and immunomodulation associated with the infections (Morrison, 1995; Karp *et al.*, 1996). Typically, CD8⁺ cells function at an early stage in virus invasion and recognize nonstructural proteins synthesized before the virus has been released from the cells. The CD4⁺ and CD8⁺ T cells may act either by interfering with virus replication through the secretion of cytokines such as interferon γ (IFN- γ) or more directly by specific MHC-linked cytotoxic activity toward virus-infected cells. The functional correlation between activation of CD8⁺ and the determination of the role of major and minor T-cell epitopes in the establishment of effective immunity constitute areas of future research.

To focus on the T-cell responses in mice we used bacterially expressed nucleocapsids to immunize mice (Jondal *et al.*, 1996). Immune splenic lymphocytes proliferate *in vitro* when restimulated with such assembled structures and the proliferation was inhibited by the addition of anti-murine MHC II antibodies. Importantly, the proliferative responses *in vitro* were not only RPV specific but also cross-reactive with PPRV, indicating that some of the CD4⁺ and CD8⁺ T-cell epitopes are conserved.

The generation of CTL-proficient in virus-infected cell killing was demonstrated by the MHC class I-restricted lysis of syngenic antigen-presenting cells presenting processed N peptides derived from the transfected cDNA or virus infection in these target cells. Once again the cross-reactivity of the N-specific lysis in immune T cells arising from RPV N- or PPRV N-immune mice con-

firms our earlier result, where we observe proliferation resulting from conserved epitopes.

We then proceeded to map a dominant H-2^d-restricted CTL epitope in BALB/c mice using a DNA construct encoding the nine amino acid conserved measles virus epitope spanning amino acids 281–289 on RPV N and PPRV N proteins. These constructs were used to transfect syngenic cells and such epitope-expressing cells were used as targets in a CTL assay. The MHC I-restricted lysis of specific cells by both RPV N- and PPRV N-immune lymphocytes proved that the sequence in question was indeed an H-2^d-restricted CTL epitope.

To demonstrate CD4⁺ and CD8⁺ T-cell responses in the natural host, we performed a proliferation assay of *in vitro* restimulated PBMC, which showed the induction of proliferation of bovine PBMC from the RBOK-RPV-vaccinated animal. Lymphocytes were isolated from a vaccinated calf at different time points postvaccination and tested for the ability to proliferate in response to *in vitro* restimulation with RPV N proteins expressed as nucleocapsid-like structures. Incorporation of radioactive thymidine indicated that the cultures were proliferating in response to soluble antigen, possibly in response to processed N peptides generated by the ingestion of the assembled nucleocapsid structures by the professional APCs present in the pool of PBMC used in the experiment (data not shown). The stimulation index was low (in the order of 2–3) but increased to 8 after a booster was administered to the bull at the end of 24 weeks p.i., indicating the generation of N-specific memory. To further assign the proliferation observed above to the CD4⁺ and CD8⁺ T-cell compartments, a proliferative assay based on antigen presentation by MHC class I⁺ and MHC class II⁺ skin fibroblasts was employed. The use of this assay demonstrated clearly the presence of CD8⁺ T cells capable of responding to the RPV N or the PPRV N as well as the H-2^d-restricted CTL epitope expressed in transfected autologous skin cells.

The results of the class I-biased proliferation assay were substantiated by employing a direct CTL assay. The CTL assay demonstrated conclusively the CD8⁺ specificity of the proliferation induced by either full-length RPV N or PPRV N or the CTL epitope identified in this study. We found that antigen-specific, MHC I-restricted killing of autologous skin cells transfected with RPV N, PPRV N, or the CTL epitope by CTLs generated in the RBOK-RPV-vaccinated bull. We used the PPRV Nigeria 75/1 strain of PPRV to infect the autologous skin fibroblasts and then used these cells to restimulate *in vitro* the PBMC and found cross-reactive CTLs in the case of all three targets used, confirming the conserved use of the nine amino acid epitope in both RPV N and PPRV N proteins.

Similar to observations made in related viruses, such as that of the generation of CD8⁺ T-cell responses in a mouse model to the adenovirus recombinant of the measles virus N protein (Fooks *et al.*, 1995) or the vaccinia

virus recombinant harboring the measles virus N protein gene (Spehner *et al.*, 1991), this study clearly establishes that CD8⁺ T cells participate in the immune response to RPV- and PPRV-infected cattle. We established a clear role for MHC I-restricted T cells in the control of virus spread and defined a CTL epitope on the N proteins of RPV and PPRV N to which T cells from immune mice and vaccinated cattle can respond. This H-2^d-restricted CTL epitope has been found to be widely conserved among all morbillivirus N proteins and experimental evidence has been provided to show the cross-reactivity to canine distemper virus N protein (Beauverger *et al.*, 1993). It will be rewarding to study the significance of this CTL epitope in a statistically larger set of animals. In light of the finding that the NP-DNA immunization failed to protect the immunized mice in the influenza system, it is of prime importance to look for alternate methods to prime the animals against the N proteins which provide a major target for virus-specific cell-mediated immunity (Childerstone *et al.*, 1999). It will also be important to see whether the use of such recombinant structures can elicit similar immune responses, especially those of cytokines such as IFN- γ and use them to study more elusive mechanisms associated with morbilliviruses such as immunosuppression and immunomodulation.

MATERIALS AND METHODS

Cells and viruses

Bovine primary skin fibroblasts were cultured from skin biopsies of the experimental animal and were maintained in IMDM supplemented with 20% FBS (fetal bovine serum) and human recombinant EGF (epidermal growth factor; Boehringer Mannheim, Mannheim, Germany) at a concentration of 10 ng/ml. These were frozen in liquid nitrogen storage for further use.

P815, a H-2^d murine mastocytoma cell line, was maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 5% heat-inactivated FBS, 50 mM L-glutamine, 0.1 mM β -mercaptoethanol, and 100 mg of streptomycin/penicillin.

The Vero cell line (obtained from National Center for Cell Science, India) was maintained in MEM (minimum essential medium) containing 5% FBS and 100 mg of strepto-penicillin.

RPV, a tissue-culture-adapted vaccine strain (RBOK, the original attenuated Kabete "O" strain of RPV) was obtained from the Institute of Animal Health and Veterinary Biologicals (Bangalore, India). Virus stocks were propagated by infection and recovery in Vero cells and subsequently titrated by TCID₅₀ assay in Vero cells as described previously (Reed and Muench, 1938).

The vaccine strain of PPRV (Nigeria 75/1), kindly provided by Dr. A. Diallo (CIRAD-EMVT, France), was grown in Vero cells and the titer was determined by TCID₅₀ assay in Vero cells (Reed and Muench, 1938).

Animals and immunization

One male calf (Jersey breed) tested negative for RPV or PPRV antibodies was vaccinated intramuscularly (im) with one dose of Tissue Culture Rinderpest Vaccine (Rinderpest vaccine strain RBOK, obtained from the Institute for the Animal Health, Pirbright Laboratory, U.K.) and boosted 6 months later) with three im doses of RPV tissue culture vaccine (made at Institute for Animal Health and Veterinary Biologicals, Bangalore).

BALB/c (H-2^d) female mice were obtained from the Central Animal Facility, Indian Institute of Science. Six- to 8-week-old mice were immunized with 25 μ g of recombinant proteins in PBS subcutaneously (sc). Serum or spleens were obtained 3 weeks postimmunization.

Antibodies

Monospecific polyclonal rabbit anti-RPV N was raised against the purified bacterially expressed RPV N protein and concentrated by 50% ammonium sulfate saturation, followed by extensive dialysis against PBS (phosphate-buffered saline). Serum from PPR disease-convalescent sheep or goats naturally infected with PPRV was used as a source of anti-PPRV antibodies.

Antibodies used for blocking of T-cell proliferation and CTL assays were obtained as a gift from Dr. D. Nandi (Dept. of Biochemistry, Indian Institute of Science). Monoclonal antibodies in the form of ascites fluid recognizing the H-2^d determinant (HB79, ATCC, Manassas, VA) and recognizing the MHC class II, I-A, and I-E determinants (TIB120, ATCC) were used at a dilution of 1:200 in the assays.

Construction of recombinant plasmids

The full-length N genes of RPV (RBOK vaccine) and PPRV (Nig 75/1 vaccine) were earlier subcloned, expressed, and characterized (unpublished results). The RPV N gene in pET20b, pSDN2 (Daniel and Shaila, 1999), and PPRV N clone in pRSET B (PGKN1) were used to transform *E. coli* BL21 (DE3) for large-scale expression.

The full-length RPV-RBOK N gene cDNA (Baron and Barrett, 1995) and the PPRV-Nig 75/1 N gene cDNA (Diallo *et al.*, 1994) were subcloned in the pCMX PL1 eukaryotic vector at the *NotI* site and *Bam*HI-*Xho*I sites, respectively. Expressions from these constructs, pCXRN and pCXPN, respectively, were assayed by immunofluorescent staining of transfected cells. The transfection was performed as described for the generation of stable transfectants described below.

The full-length RPV-RBOK N gene cDNA and the PPRV-Nig 75/1 N gene cDNA were subcloned in the pCDNA3 eukaryotic vector (Invitrogen, San Diego, CA) in the multiple cloning site at the *NotI* and the *Eco*RI-*NotI* sites, respectively, for stable expression in eukaryotic cells. The pCDNA3 series of vectors have a neomycin-resis-

tance cassette in addition to the genetic elements required for propagation in both prokaryotic and eukaryotic hosts.

The full-length pCDNA3 constructs pSRN6^{neo} and pSPN6^{neo} were used to transform *E. coli* DH5 α for isolation of plasmid DNA for stable transfection.

Synthetic oligonucleotides were designed and synthesized (Bangalore Genei, India) as minigenes (Schirmbeck *et al.*, 1995) encoding nine amino acids of a predicted CTL epitope YPALGLHEF (Beauverger *et al.*, 1993, 1996), which corresponds to amino acids 281–289 on both RPV N and PPRV N proteins and were flanked by noncomplementary restriction sites *Bam*HI and *Pst*I represented as underlined bases: {oligo N1 5'-ACG-GGATCCTACCCAGCACTGGGCCTGCATGAATTCACT-GCAGCCAA-3'; oligo N2 5'-TTGGCTGCAGTGAATTC-ATGCAGGCCCACTGCTGGGTAGGGATCCGTT-3'}. The oligonucleotides were annealed, digested with the restriction enzymes to generate sticky ends, and ligated directionally into pRSET B. The recombinant clones were confirmed by colony hybridization with the radiolabeled N1 oligonucleotide. A positive clone was then used to release the minigene along with the vector transcription start site and stop signal. This released fragment was further ligated directionally into the eukaryotic expression vector pCMX.PL2 containing CMV immediate-early gene promoter, f1 origin of replication, SV40 origin of replication and promoter, and a multiple cloning site. Recombinant clones were confirmed by colony hybridization with N1 oligo probe, and a single positive colony was selected (pSM2) and used to prepare DNA for all transfection experiments. The schematic representation of the generation of the synthetic CTL epitope construct is shown in Fig. 5. DNA for all transfections were prepared in prepacked columns Qia-tip 100 (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

Generation of stable transfectants

P815 cells were seeded on 60-mm tissue culture petriplates and transfected with 5–10 μ g each of the pSRN6^{neo} or pSPN6^{neo} constructs in OPTIMEM (Life Technologies) using lipofectamine for 12 h. After 12 h the transfection medium was removed and replaced by normal RPMI 1640 containing 5% FBS for 72 h. G418 (geneticin) was added to these cultures at a concentration of 400 μ g/ml and the resistant cells were screened for expression of the RPV and PPRV N proteins 3 weeks after antibiotic selection was applied. The clones were selected by single-cell cloning in the presence of the antibiotic by the limiting dilution method. The expression from these cells was assayed by immunofluorescent staining of stably transfected clones. The positive clones were maintained in 100 μ g/ml of G418 (Life Technologies).

Purification of antigens

E. coli BL21(DE3) cells transformed with pSDN2 (RPV N) or pGKN1 (PPRV N) were grown in 500 ml LB medium and induced with 0.4 mM IPTG for 5 h. Harvested cells were resuspended in Buffer A containing 50 mM Tris-HCl, pH 8, 7.5% glycerol, 0.1 mM EDTA, 1 mM DTT, and 50 mM NaCl. The cells were then lysed on ice for 1 h by the addition of Buffer B containing 50 mM EDTA, 10% Triton X-100, and 2.5 mg/ml lysozyme. The cells were sonicated briefly after the addition of 5 mM MgCl₂ and the lysate was clarified by a high-speed spin at 13,000 rpm for 30 min at 4°C. The nucleocapsids were purified as described earlier (Daniel and Shaila, 1999). Briefly, 1–2 ml of the cleared lysates were layered over a 20, 30, and 40% (w/v) step CsCl gradient prepared in PBS and centrifuged in a SW41 rotor at 37,000 rpm in a Beckman ultracentrifuge (Beckman Coulter, Fullerton, CA) for 16 h at 4°C. The NC band at the interface of the 30 and 40% layers was collected and diluted in 10 volumes of PBS and further pelleted in a Ti-70 Beckman rotor at 43,000 rpm for 1 h at 4°C. The pellet obtained was resuspended in 400 μ l of PBS. The protein content was estimated by Bradford's method and the purity was checked by Coomassie staining of samples run on SDS-PAGE.

Electron microscopy

Purified nucleocapsid proteins were resuspended in PBS at a concentration of 250 μ g/ml. The NC particles were visualized by placing the sample over a copper grid coated with carbon film and allowed to adsorb for 2 min. The grid was subsequently washed with distilled water for 30 s and negatively stained with 2% potassium phosphotungstate, pH 7.0, for 1 min. The grid was air-dried at room temperature for 10–15 min and examined in a Jeol 100cX11 electron microscope at 80 kV.

Lymphocyte proliferation assays with soluble antigens

Lymphocytes were prepared from the immune spleen and purified on a Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient by centrifugation at 3000 rpm for 30 min. The buffy coat layer was recovered, washed with RPMI, and resuspended in RPMI containing 10% FBS. A total of 1×10^4 splenic lymphocytes were plated per well and incubated with different concentrations of the soluble RPV or PPRV N proteins in a final volume of 200 μ l/well for 5 days. Tritiated thymidine (0.5 mCi/well; spec. act. 6500 mCi/mmol; BARC, Mumbai, India) was added 16 h before harvesting the cultures. The incorporated radioactivity was measured in a scintillation spectrometer.

CTL assays in the murine system

Three weeks after the immunization with purified nucleocapsids, splenic lymphocytes were harvested as de-

scribed above and restimulated *in vitro* with 2 μ g of purified RPV or PPRV nucleocapsids. After 5 days CTL activity was assessed using P815 cells infected with RBOK RPV or PPRV Nig 75/1 or P815 cells transfected with pCXRN or pCXPN or stably transfected P815 cells (described above) or CTL epitope-expressing cells (pSM2 transfected) as target cells. CTL activity was measured by a CTL detection kit (Boehringer Mannheim) as described in the manufacturer's instructions. The percentage specific lysis was calculated by the formula

% specific lysis

$$= 100 \times \frac{(\text{experimental} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})}$$

Bovine MHC class I-biased lymphocyte proliferation assays

Autologous primary skin fibroblast cells obtained from the experimental animal before vaccination (see above) were transfected with 10 μ g of the plasmid constructs containing the full-length RPV N (pCXRN) or PPRV N (pCXPN) or the pSM2 DNA and 24 h after transfection cells were gamma-irradiated at 3500 rads for 3 min before use as CTL targets. Target cells were plated in a 96-well flat-bottom tissue culture plate (Nalge Nunc, Rochester, NY) in different numbers ranging from 2000 to 8000 cells/well. Bovine PBMC were isolated from 1:2-diluted heparinized blood and purified on Ficoll-Hypaque as above. The buffy coat was recovered, washed with RPMI, and resuspended in RPMI containing 10% FBS. PBMC (1×10^5) were added to the irradiated stimulators in a final volume of 200 μ l and incubated in a humidified CO₂ incubator at 37°C for 5 days. Control assays were set up using untransfected fibroblasts as stimulators. Tritiated thymidine (0.5 mCi/well) was added 16 h before harvesting the cultures. The incorporated radioactivity was measured in a scintillation spectrometer. The stimulation index (SI) was calculated by:

$$\text{SI} = \frac{\text{mean cpm of triplicate antigen-stimulated wells}}{\text{mean cpm of triplicate control wells}}$$

Bovine cytotoxic T-lymphocyte (CTL) assays

Autologous primary skin fibroblast cells (1×10^6) were transfected with 10 μ g of the pCXRN, pCXPN, or the pSM2 plasmid DNA using lipofectamine (Life Technologies) for 24 h. Cells were gamma-irradiated at 3500 rads for 3 min. Cells were plated in a 96-well flat-bottom tissue culture plate (Nalge Nunc). PBMC isolated as described earlier were added at different ratios to these targets in a final volume of 200 μ l and incubated in a humidified CO₂ incubator at 37°C for 12 h. Control assays were set up using uninfected fibroblasts as targets. The presence of CTL activity was assessed in triplicate experiments.

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